

Charles University
Faculty of Science

Study programme: Biology
Branch of study: Cellular and Developmental Biology



Bc. Daniela Hávová

Analysis of casein kinase γ function in model organism *Caenorhabditis elegans*
Analýza funkce kasein kinázy γ v modelovém organismu *Caenorhabditis elegans*

Diploma thesis

Supervisor: Mgr. Marie Macůrková, Ph.D.

Prague, 2020

Tímto bych chtěla poděkovat svojí školitelce Mgr. Marii Macůrkové, PhD. bez jejíž trpělivosti a odborné pomoci by tato práce nevznikla. Také bych chtěla poděkovat všem členům Laboratoře molekulární genetiky vývoje na Univeritě Karlově, zejména Mgr. Lence Doubravské, PhD., Mgr. Filipu Knopovi a Mgr. Jitce Velčevové za trpělivost, vlídnost a pomoc při experimentech.

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Kunžaku, 14.4.2020

Bc. Daniela Hávová

Abstract

Casein kinase 1 (CK1) protein family is an important part of signalling apparatus in eukaryotic organisms. These serin-threonine kinases play roles in several important developmental and cell-maintaining signalling pathways including Wnt signalling. CK1 kinases influence this signalling pathway in various manners; some of them positively – leading to expression of Wnt regulated genes and some of them negatively – contributing to degradation of the transcription activator β -catenin.

In this thesis, we were focusing on casein kinase 1 gamma (CK1 γ), which in mammalian cells in the presence of Wnt signal phosphorylates Wnt co-receptor LRP5/6. This phosphorylation leads to binding and inhibition of a β -catenin degradation complex. In *C. elegans* LRP5/6 receptor is probably missing from the genome, yet we can still observe influence of CSNK-1/CK1 γ on Wnt signalling. Using reduction-of-function experiments we have found out that loss of *csnk-1* expression enhanced a canonical Wnt signalling defect in mutants with reduced Wnt production. Using overexpression approach, we were not able to uncover the tissue specificity of CSNK-1 action. In order to obtain a better tool to study the function of CSNK-1, we began to optimize conditions for the use of auxin-inducible degron system for conditional CSNK-1 depletion.

Abstrakt

Rodina kasein kináz 1 (CK1) je důležitou součástí signalizačního aparátu všech eukaryotických organismů. Tyto serin-threoninové kinázy se zapojují do několika signálních drah důležitých pro vývoj a udržování buněčných tkání, včetně Wnt signalizace. Tuto dráhu ovlivňují kinázy CK1 rodiny různými způsoby, některé pozitivně – vedou signalizaci k expresi Wnt řízených genů a některé negativně – napomáhají degradaci transkripčního aktivátoru β -catenin.

V této práci jsme se zaměřili na kasein kinázu 1 gamma (CK1 γ), která v savčích buňkách za přítomnosti Wnt signálu fosforyluje Wnt koreceptor LRP5/6. Tato fosforylace následně způsobuje vazbu a inhibici degradačního komplexu β -catenin. U *C. elegans* tento LRP5/6 koreceptor v genomu pravděpodobně chybí, přesto však můžeme pozorovat, že CSNK-1/CK1 γ Wnt signalizaci ovlivňuje. Za pomoci experimentů redukujících funkci jsme zjistili, že ztráta exprese CSNK-1 zesiluje defekty v kanonické Wnt signalizaci u mutantů se sníženou produkcí Wnt molekuly. Metodou overexprese jsme nebyli schopni odhalit tkáňovou specifitu působení CSNK-1. Abychom získali lepší nástroje pro studium funkce CSNK-1, začali jsme s optimalizací podmínek pro auxinem indukovaný degradační systém pro podmíněnou degradaci CSNK-1.

Content

List of abbreviation.....	1
1 Introduction.....	4
1.1 <i>Caenorhabditis elegans</i> as a model organism.....	4
1.2 Wnt signalling.....	6
1.3 Wnt signalling in <i>C. elegans</i>	7
1.4 QL neuroblast migration	9
1.5 CSNK-1 as member of the CK1 protein family.....	11
1.6 CK-1 γ in other organisms	13
1.7 Auxin inducible degron.....	14
1.7.1 Principle.....	14
2 Objective.....	16
3 Materials and methods.....	17
3.1 Strains.....	17
3.1.1 Bacterial strains	17
3.1.2 <i>C. elegans</i> strains and transgenes.....	17
3.2 Cultivation media.....	17
3.2.1 Bacteria cultivation media.....	17
3.2.2 <i>C. elegans</i> cultivation.....	18
3.2.3 <i>C. elegans</i> bleaching.....	19
3.3 RNAi experiments.....	19
3.3.1 RNAi mechanism	19
3.3.2 RNAi plates	20
3.3.3 RNAi bacteria.....	20
3.3.4 RNAi experiment.....	20
3.4 <i>csnk-1</i> cloning.....	20
3.4.1 Isolation of RNA	20
3.4.2 cDNA synthesis.....	21
3.4.3 pJet cloning and ligation	22
3.4.4 Bacterial transformation	23
3.4.5 Gibson assembly.....	23
3.4.6 Injection of plasmids.....	25
3.5 Early QL migration experiments.....	26

3.6	AID system.....	27
3.6.1	Auxin plates.....	27
3.7	Microscopy and image editing.....	27
3.7.1	Worm preparation for microscopy.....	27
3.7.2	Microscopy.....	27
3.7.3	Image editing.....	27
3.8	Others.....	28
3.8.1	Electrophoresis in agarose gel.....	28
3.8.2	Plasmid DNA isolation.....	28
4	Results.....	29
4.1	RNAi.....	29
4.2	Overexpression.....	30
4.3	Early QL polarization.....	31
4.4	AID system.....	33
4.4.1	Auxin experiments.....	33
5	Discussion.....	36
5.1	RNAi experiments.....	36
5.2	Overexpression.....	38
5.3	Early QL migration.....	40
5.4	AID system.....	41
6	Literature.....	43

List of abbreviation

AID	auxin inducible degron
APC	adenomatous polyposis coli
AQR	<i>C. elegans</i> sensory neuron
Arrow	<i>Drosophila</i> homolog protein for human LRP5/6
AVM	<i>C. elegans</i> sensory neuron
BAR-1	<i>C. elegans</i> ortholog for human β -catenin
Cas9	CRISPR associated protein 9
CK1	casein kinase 1
CRISPR	method for gene editing
CSNK-1	<i>C. elegans</i> Casein kinase 1 gamma
CWN-1	<i>C. elegans</i> ortholog for WNT
CWN-2	<i>C. elegans</i> ortholog for WNT
DEPC H ₂ O	nuclease-free water
DH5 α	<i>E. coli</i> strain competent for plasmid cloning
DIC	differential interference contrast microscopy
dNTP	deoxynucleotide triphosphate
DPY-19	<i>C. elegans</i> ortholog of human DPY19L
DTT	dithiotreitol
Dvl	Dishevelled protein
<i>Eft-3</i>	gene for eukaryotic translation elongation factor
EGL-17	egg laying defective protein, <i>C. elegans</i> ortholog for human FGF17
EGL-20	<i>C. elegans</i> ortholog of human WNT
FZ	Frizzled receptor
GISH	Gilgamesh
GSK-3 β	glucogen synthase kinase 3 β
HEK293T	human embryonic kidney cell line
HMP-2	<i>C. elegans</i> beta-catenin-like protein

HMR-1	<i>C. elegans</i> cadherin-related protein
HT115	<i>E. coli</i> strain for RNAi feeding
IPTG	isopropyl β -D-1-thiogalactopyranoside
L1-L4	Four larval stadia of <i>C. elegans</i>
L4440	vector for RNAi
LEF	lymphoid enhancer-binding factor
LIN-17	<i>C. elegans</i> ortholog of human FZD4
LIN-44	<i>C. elegans</i> ortholog of human LRP5/6
MAB-5	<i>C. elegans</i> ortholog for human homeobox 6A protein
MIG-1	<i>C. elegans</i> ortholog for Fz
MIG-14	<i>C. elegans</i> ortholog for Wls
MIG-21	<i>C. elegans</i> receptor
MIG-5	<i>C. elegans</i> ortholog for human Dvl
MOM-2	<i>C. elegans</i> ortholog for Wnt
MOM-5	<i>C. elegans</i> ortholog for Fz
MTM-6/9	myotubularin related proteins
NF-AT4	nuclear factor of activated T-cells, transcription factor
OP50	<i>E. coli</i> strain for common <i>C. elegans</i> feeding
PCP	planar cell polarity pathway
POP-1	<i>C. elegans</i> ortholog of human LEF1
PQR	<i>C. elegans</i> sensory neuron
PTP-3	<i>C. elegans</i> ortholog of human PTPRD
PVM	<i>C. elegans</i> sensory neuron
QL	left Q neuroblast
QL.d	QL descendants
QR	right Q neuroblast
ROR	tyrosine-protein kinase transmembrane receptor
RRF-3	<i>C. elegans</i> RNA dependent RNA polymerase
RT-PCR	reverse transcription PCR

SDQL	<i>C. elegans</i> sensory neuron
SDQR	<i>C. elegans</i> sensory neuron
SID-1/2	RNA transmembrane transporters
siRNA	small interfering RNA
SLS	Ser, Lys, Ser motif
SNX-3	sorting nexin
TEF	thyrotroph embryonic factor
TGF- β	transforming growth factor β
TIR1	F-box protein
UNC-40	<i>C. elegans</i> ortholog for human DCC and NEO1
UNC-54	<i>C. elegans</i> ortholog for MYH1
V1-6	seam cells
VPS-29	retromer complex component
β -TrCP	Beta-transducin repeats-containing proteins

1 Introduction

1.1 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans is one of the model organisms used for laboratory experiments. Its small size (adult approx. 1.5 mm) and short lifecycle (around 3 days) allow growing hundreds of animals in few days in a small space. Further, the cultivation is convenient and inexpensive. Worms are fed on *E. coli* growing on agar plates at the optimal growing temperatures between 12-25°C (Corsi *et al.*, 2015).

C. elegans has two sexes: self-fertilizing hermaphrodite, containing two X chromosomes (XX), and male containing one X chromosome (X0). The ratio of X to autosomes initiates sex determination (Zarkower, 2006). Male frequency is between 0.1-0.2 % making them quite rare but important for maintaining diversity. We can use these for introducing mutations to a certain strain by crossbreeding (Corsi *et al.*, 2015).

Hermaphrodites are a great tool for maintaining strains with a specific genotype as they form the major part of progeny since the meiotic non-disjunction of X chromosomes leading to male progeny is very rare. A single worm can populate a plate with genetically identical animals in a short time. Similarly it is also possible to grow *C. elegans* in liquid culture for a limited time (Lewis and Fleming, 1995; Corsi *et al.*, 2015).

The life cycle of *C. elegans* slows with decreasing temperature. In extreme conditions such as starvation, dauer larva is formed as a survival mechanism. Worms can survive for several months in this state at 16 °C which is a convenient feature for maintaining the most used strains. After transferring to a fresh plate with *E. coli*, worms will resume their normal life cycle. For long-term stocks, it is possible to freeze worms with a cryoprotective agent and by slow freezing conditions. Frozen worms can be stored at -70°C for years (Brenner, 1974; Lewis and Fleming, 1995).

C. elegans is translucent, so easily observable under the microscope and it has an invariant number of cells. Combination of these properties allows us to spot a single cell of interest and follow it during the development in a living animal. Some phenotypes are observable under a stereomicroscope, for most of the other observation under a compound microscope is sufficient. Therefore, *C. elegans* doesn't require specific expensive tools to

work with (Sulston and Horvitz, 1977; Silhankova and Korswagen, 2007; Corsi *et al.*, 2015).

The foundation of *C. elegans* genetics was laid by Brenner and Sulston team, who invented a lot of methods for nematodes handling and performed the first large-scale mutant screens on it and who were able to describe all somatic cell lineages in *C. elegans* (Sulston and Brenner, 1973; Brenner, 1974; Sulston and Horvitz, 1977; Boulin, 2012). In 1991 Mello *et al.* showed the mechanism of DNA transformation using gonadal microinjection followed by Chalfie *et al.* discovery of GFP marker for gene expression and protein visualisation in 1994 (Mello *et al.*, 1991; Chalfie *et al.*, 1994). Another significant year for *C. elegans* was 1998 when the whole genome was sequenced as the first multicellular organism in the world and Fire *et. al* introduced mechanism of RNA interference by double-stranded RNA, another tool for genetic screens (Fire *et al.*, 1998). All of these methods, with slight modifications, are still in use today and work very effectively for *C. elegans* research.

The relevance of *C. elegans* model system for human biology and disease modelling can be discussed based on similarities in the proteome, signalling and developmental pathways. First protein comparisons began to emerge in 1997. For 60% of then known *H. sapiens* proteins homologs were found in *C. elegans* unfinished genome with the prediction increasing the number to 70% (Sonnhammer and Durbin, 1997). A list of 70 positionally cloned human disease genes were searched for within the *C. elegans* genome and 40% of very close matches were found (Ahringer, 1997). This was confirmed by Culetto *et. al* in 2000 with 42% of matches (Culetto, 2000). In 2011, Ortholist based on four programmes for orthology prediction was made including orthologs for fundamental signalling pathways as Notch, Wnt, TGF- β , insulin signalling pathways and families of proteins including protein kinases, F-box proteins, nuclear hormone receptors and transcription factors. Nematode kinases, drivers for most of the signalling functions, show 81 % of homology for all human kinases (Manning, 2005; Shaye and Greenwald, 2011). Such findings justify the use of *C. elegans* as a model for studying basic mechanisms of signal transduction and development.

1.2 Wnt signalling

Wnt signalling is one of the most important developmental and homeostatic pathways. It plays a role in cell differentiation, cell polarity, cell migration, stem cells maintaining and other processes. Deregulation of this pathway is leading to severe disorders, including cancer. It is a complex pathway with numerous regulations and crosstalk (Clevers and Nusse, 2012; Niehrs, 2012; Nusse and Clevers, 2017). Wnt signalling pathways are usually divided into β -catenin dependent (canonical) and β -catenin independent (non-canonical) pathways. From non-canonical pathways, we can label for example, the PCP (planar cell polarity) pathway, which uses Frizzled receptor, although other components are different from the canonical pathway, or RYK and ROR receptors dependant pathways (Kikuchi *et al.*, 2011). However, this thesis will focus on the most studied pathway, the canonical Wnt/ β -catenin signalling pathway.

Main molecular outcome of Wnt/ β -catenin canonical pathway is the prevention of destruction of β -catenin transcription coactivator and initiation of expression of Wnt regulated genes. Wnt signalling in mammals starts with the secretion of Wnt molecule from the producing cell, accessing the receiver cell from the extracellular side. This molecule is binding to the receptor Frizzled (Fz) and coreceptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) creating the signalosome complex. Casein kinase 1 γ (CK1 γ) phosphorylates LRP5/6 on C-terminal tail inside the cell, which leads to recruitment of Axin and Dishevelled (Dvl) protein (Del Valle-Pérez *et al.*, 2011). This leads to the binding and inhibition of the destruction complex on the membrane. The destruction complex is composed of two scaffold proteins – adenomatous polyposis coli (APC) and Axin and two kinases – glycogen synthase kinase 3 β (GSK-3 β) and CK1 α . In the absence of Wnt signal, this complex is binding β -catenin and sending it for destruction in the proteasome. When the Wnt signal is present, transcription regulator β -catenin is accumulating in the cytoplasm, enters the nucleus and influences TEF/LEF transcription factors towards the expression of Wnt controlled genes (Sawa and Korswagen, 2013; Cruciat, 2014).

The key event of Wnt/ β -catenin signalling is GSK-3 β inhibition. GSK-3 β is a kinase named after its function in glycogen metabolism, but the range of its substrates is much wider. One of these substrates is transcriptional coactivator β -catenin, which bound in

destruction complex, is phosphorylated by GSK3- β and CK1 α kinase on multiple sites. These phosphorylated sites are then recognized by β -TrCP E3 ubiquitin ligase, ubiquitinated and sent for destruction in the proteasome (Liu *et al.*, 2002; Del Valle-Pérez *et al.*, 2011).

Phosphorylation is an important modification for signal transmission. CK1 protein family consisting of six members plays an important role in Wnt canonical pathway. Roles in this pathway could be both activating and inhibiting depending on given circumstances and behaviour of the particular member of the family. These activities are very complex with cross-talks, overlaps and contradictions (Cruciat, 2014).

1.3 Wnt signalling in *C. elegans*

Components of the Wnt canonical signalling are conserved in *C. elegans* (shown in Figure 1) with the possible exception for LRP6/Arrow coreceptor, which has not been found yet (Sawa and Korswagen, 2013).

C. elegans encodes five WNT molecules in its genome: *cwn-1*, *cwn-2*, *lin-44*, *egl-20* and *mom-2*, from which *egl-20* is the one participating in WNT canonical signalling (Herman *et al.*, 1995; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Maloof *et al.*, 1999; Zinovyeva *et al.*, 2008). These molecules have a dual character acting either as short range signals or as a long range concentration gradient creating guidance cue for cell migration (Zecca *et al.*, 1996). *C. elegans* Wnts are playing roles in various events during the development of the animal, for example, neuronal cell migration, asymmetric cell division or cell polarization (Sawa and Korswagen, 2013). Each WNT is influencing at least one neuron migration, but CWN-1, CWN-2 and EGL-20 have the most prominent effect. Mutations in these WNTs are causing most of serious cell migration defects (Zinovyeva *et al.*, 2008).

There are four Frizzled receptors in *C. elegans*, but canonical Wnt signalling can be triggered only by three of them – MIG-1, LIN-17 and MOM-5 (Harris *et al.*, 1996). The last Frizzled, CFZ-2, is involved in several non-canonical pathways (Zinovyeva and Forrester, 2005; Song *et al.*, 2010). Also, three proteins from Dishevelled family are present – DSH-1, DSH-2 and MIG-5. MIG-5 is the one contributing to the Wnt pathways, and together with the other two is active in spindle positioning (Korswagen *et al.*, 2002; Walston *et al.*, 2004).

C. elegans has also an analog of the destruction complex. Proteins PRY-1 and AXL-1 despite very low similarity in sequence were found to be functional homologs of mammalian Axin. Both proteins are interacting with BAR-1/ β -catenin, SGG-1/GSK-3 β and MIG-5/Dvl. Only PRY-1 interact with APR-1/APC-like protein and they likely function together in canonical pathway (Maloof *et al.*, 1999; Gleason *et al.*, 2002; Korswagen *et al.*, 2002; Oosterveen *et al.*, 2007).

Whereas *Drosophila* has one and mammals have two β -catenin homologs for both signalling and structural functions, *C. elegans* has four of them – BAR-1, WRM-1, SYS-1 and HMP-2 (Butz *et al.*, 1992; Orsulic and Peifer, 1996; Korswagen *et al.*, 2000; Kidd *et al.*, 2005). BAR-1 is the main β -catenin homolog interacting with Tcf/POP-1 transcription factor to start expression of the Wnt regulated genes in the canonical pathway (Eisenmann *et al.*, 1998; Gleason *et al.*, 2002; Korswagen *et al.*, 2002). HMP-2 is an exclusive β -catenin for adherens junctions, where it binds HMR-1 cadherin. The other two β -catenins have also Tcf/POP-1 binding activity and they are acting in embryogenesis and during certain asymmetric cell divisions. WRM-1 was shown to regulate levels of POP-1 in the nucleus and SYS-1 is coactivator of Wnt regulated genes (Huang *et al.*, 2007; Phillips *et al.*, 2007).

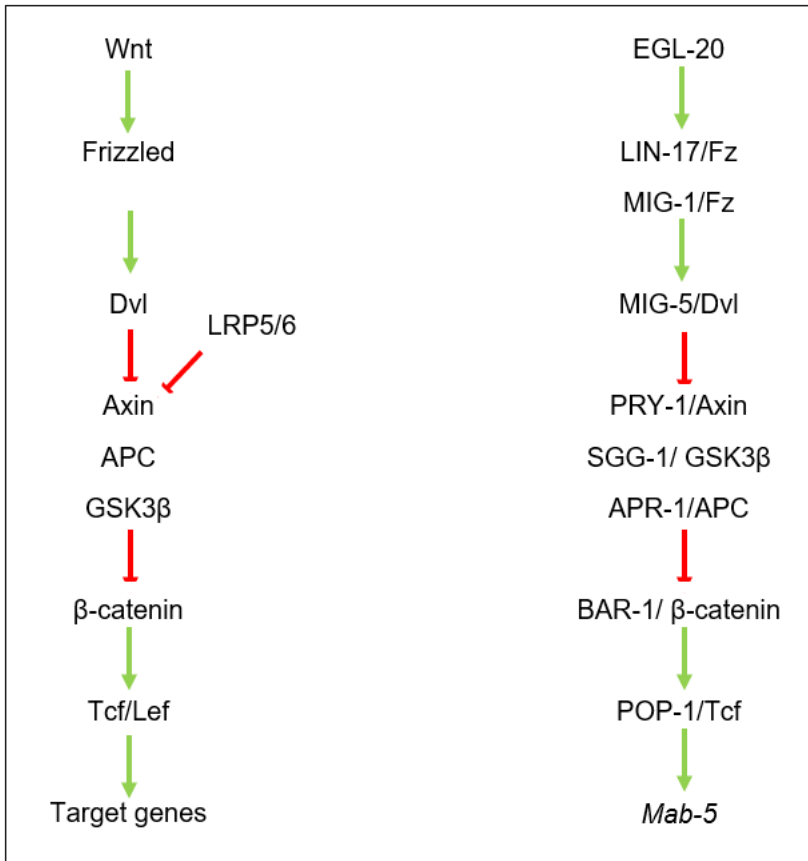


Figure 1: Scheme of canonical Wnt signalling pathway components in mammals (on the left) and *C. elegans* (on the right).

1.4 QL neuroblast migration

In the late embryonal and early first larval stage of *C. elegans*, several different neurons migrate along the anterior-posterior body axis. Two of these neurons are the QL and QR neuroblasts and their descendants migrating to their final position in the worm body (Sulston, 1977; Silhankova and Korswagen, 2007).

Q neuroblasts are born symmetrically on lateral sides in the posterior last third of the worm between V4 and V5 seam cells. QL (on the left side) and QR (on the right side) are not only symmetrical in their initial positions but also in their division patterns. The difference lies in opposite directions of migration of their progeny (Sulston and Horvitz, 1977; Honigberg and Kenyon, 2000).

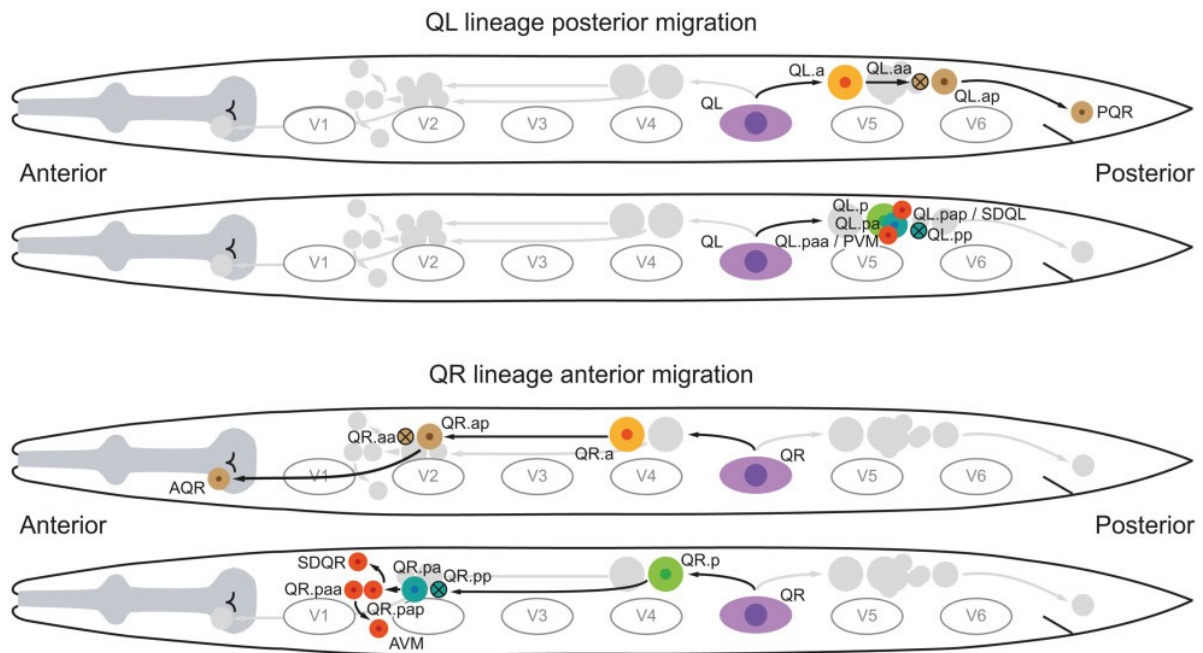


Figure 2: Scheme of Q neuroblasts divisions and migration. Q neuroblasts (violet ovals) are born symmetrically on the lateral sides between V4 and V5 seam cells (white ovals V1-V6). From the ventral view, QR is on the right side of the animal, QL on the left side. Upper two worm schemes are showing division and migration of QL descendants QL.a and QL.p and their daughters. On the lower two schemes is the fate of QR.p and QR.a descendants from QR neuroblast. Both lineages have the same dividing pattern, but the descendants migrate to the opposite sides of the worm. Taken from (Rella *et al.*, 2016)

At first, Q neuroblasts are migrating short distance over the adjacent seam cells. QL migrates over the V5 seam cell posteriorly, QR migrates over V4 seam cell anteriorly. In this position, Q cells are initiating the first symmetrical division into Q.p and Q.a daughter cells. These cells are undergoing further migration and division to form sensory neurons PVM and PQR, AVM and AQR and also interneurons SDQR and SDQL. The scheme of Q cells divisions and migration is in Figure 2 (Sulston and Horvitz, 1977; Honigberg and Kenyon, 2000; Middelkoop and Korswagen, 2014).

Migration of the Q neuroblasts is driven by several mechanisms. For polarisation and short-range migration from the initial position over adjacent seam cells, PTP-3/PTPRD phosphatase, UNC-40/DCC netrin receptor and two transmembrane proteins DPY-19/DPY-19L and MIG-21 are necessary, but the exact mechanism of their action is not known yet (Honigberg and Kenyon, 2000; Middelkoop *et al.*, 2012; Sundararajan and Lundquist, 2012).

After this initial migration QL neuroblast becomes sensitive to the Wnt molecule EGL-20 and initiates Wnt signalling which leads to expression of the Hox gene *mab-5* and further

posterior migration. At the same time QR is not sensitive to the level EGL-20 present, and as a consequence does not activate canonical pathway and is continuing in anterior migration due to no *mab-5* expression (Rella *et al.*, 2016).

The importance of the EGL-20/Wnt pathway for proper QL migration is underlined by the fact that not only components of the Wnt transduction pathway but also components of the Wnt production machinery are required for correct migration of QL. In *C. elegans*, EGL-20/Wnt is produced in cells located around the rectum – P9/10, K, F, U, B, mu anal cells (Whangbo and Kenyon, 1999). In order to be secreted, EGL-20 requires several molecules. First, on the way out of the producing cells EGL-20 is bound to MIG-14/Wntless receptor that helps to transport it from the Golgi apparatus to the cytoplasmic membrane (Bänziger *et al.*, 2006). After EGL-20 release on the membrane, MIG-14 is recycled by the action of the retromer complex components VPS-26, VPS-29 and VPS-35 (Coudreuse *et al.*, 2006; Prasad and Clark, 2006; Yang *et al.*, 2008) and with the help of myotubularin lipid phosphatases MTM-6 and MTM-9 (Silhankova *et al.*, 2010).

If any of the components of the canonical pathway is disrupted, the QL neuroblast does not start its posterior migration and instead its descendants migrate to the anterior in a similar manner as the descendants of the QR neuroblast. This reversal of migration will be in this thesis referred to as the “QL phenotype”. We can observe this phenotype in mutations of all the above mentioned canonical Wnt signalling genes – *mab-5/homeobox protein A6/B6*, *bar-1/β-catenin*, *pop-1/Tcf*, *egl-20/Wnt*, *mig-14/WLS*, *mig-1/Fz*, *lin-17/Fz*, *mig-5/Dvl*, *vps-35*, *vps-29*, *vps-26* (Salser and Kenyon, 1992; Harris *et al.*, 1996; Maloof *et al.*, 1999; Korswagen *et al.*, 2002; Coudreuse *et al.*, 2006; Walston *et al.*, 2006).

Finally, once the direction of migration is established in both the QL and QR descendants, several non-canonical Wnt pathways are sequentially activated and those control the final position where the cells will stop their migration. All five Wnts are involved in these subsequent migrations (Zinovyeva *et al.*, 2008; Rella *et al.*, 2016).

1.5 CSNK-1 as member of the CK1 protein family

CK1 family of kinases was discovered in the 1970s accidentally during the experiments with AMP-dependent kinases making them among the first described protein kinases. Kumar and his team found out, that these kinases can phosphorylate casein independently of AMP molecule, but with the presence of ATP or GTP. The exact function

of these kinases was unknown, thus this family got the name casein kinase (Kumar and Tao, 1975).

Later was shown that these kinases are conserved in eukaryotes from yeasts to human. This family of Ser/Thr protein kinases consists of six members in human: α , $\gamma 1$, $\gamma 2$, $\gamma 3$, δ and ϵ . Sometimes, these are labelled as isoforms, even though they are coded by different genes. However, after post-transcriptional modifications, we can find a huge number of splicing variants, for example, four splicing variants of CK1 α with different cellular and biochemical properties (Burzio *et al.*, 2002; Cheong and Virshup, 2011).

CK1 protein family has a similar structure to other kinases with smaller N-lobe, larger C-lobe with ATP binding site and catalytic cleft in between. The most conserved part of the proteins within the family is the kinase domain with identity between 53-98 %. On the contrary, most of the variability between isoforms is in the C-terminal domain carrying specificity and regulation sequences (Cheong and Virshup, 2011).

CK1 γ is unique in this family with C-terminal motifs (TKCCFFKR), which can be possibly modified with palmitoylation, anchoring the protein to the plasmatic membrane. This assumption was confirmed by Davidson *et al.*, 2005, who was able to observe CK1 γ on the membrane in *Xenopus* and human HEK293T cells and showed cytoplasmatic localization of CK1 γ missing these motifs (Davidson *et al.*, 2005).

The preferred site for CK1 phosphorylation is already phosphorylated one (primed) or with basic residue on position N-3 such as D/pT/pS-X-X-S/T, where p is phosphorylation, X any AA and the last underlined serine/threonine is the site that will be phosphorylated by CK1. Kinase is using this primed site or acidic residue for proper positioning of the target site to the catalytic cleft (Meggio *et al.*, 1992; Cheong and Virshup, 2011). However, this consensus sequence is missing in some substrates phosphorylated by CK1. One of these substrates is the already mentioned β -catenin, primarily phosphorylated by CK1 α , APC or NF-AT4. These proteins were sequentially compared and some other common features such as SLS (Ser, Lys, Ser) motif and acidic cluster were discovered, which could determine CK1 specificity in these cases (Marin *et al.*, 2003).

The same behaviour was observed in *Drosophila* in phosphorylation of Smoothened. This G-protein-coupled receptor, participating in Hedgehog signalling, needs to be primed by protein kinase A before the Gilgamesh/CK1 γ phosphorylation (Li *et al.*, 2016).

Very limited amount of data is so far available about CSNK-1, the *C. elegans* ortholog of CK1 γ . Only two reports bring some information about its function. First, Panbianco and colleagues studied its activity in one cell embryo. They found out that CSNK-1 acts downstream of PAR proteins and regulates asymmetric localization of another kinase, phosphatidylinositol 4-phosphate 5-kinase PPK-1 leading to asymmetric spindle positioning. They also found that CSNK-1 is localizes to the plasmatic membrane confirming this property of CK1 γ enzymes also in *C. elegans* (Panbianco *et al.*, 2008). However this study did not show whether PPK-1 is directly phosphorylated by CSNK-1. The second study described a role of CSNK-1 during meiosis. Loss of *csnk-1* expression resulted in formation of large polar bodies during meiotic division and the reason was that the actomyosin contractile ring was incorrectly positioned during the division (Flynn and McNally, 2017). The direct target of CSNK-1 phosphorylation was also not identified in this work.

1.6 CK-1 γ in other organisms

So far, the best description of CK1 γ function comes from mammals and *Xenopus*, where this kinase is involved primarily in the canonical Wnt/ β -catenin pathway. Here CK1 γ is phosphorylates Wnt coreceptor LRP5/6 on its cytoplasmic domain (Davidson *et al.*, 2005; Zeng *et al.*, 2005). Davidson and colleagues showed that LRP6 without N-terminal extracellular domain is still able to bind CK1 γ , while LRP6 with missing intracellular part is not. They went even further and identified exact clusters for CK1 phosphorylations and binding sites for Axin protein (Figure 3, Davidson *et al.*, 2005). The ability of CK1 γ to phosphorylate LRP5/6 was shown also in *Drosophila* (Zhang *et al.*, 2006).

However, *Drosophila* CK1 γ ortholog called Gilgamesh (Gish) takes part also in other pathways as the Hedgehog pathway where Gish is phosphorylating the G protein coupled receptor family protein Smoothened (Li *et al.*, 2016). Gish also participates on regulation of the non-canonical Wnt-PCP pathway by interfering with vesicle trafficking (Gault *et al.*, 2012).

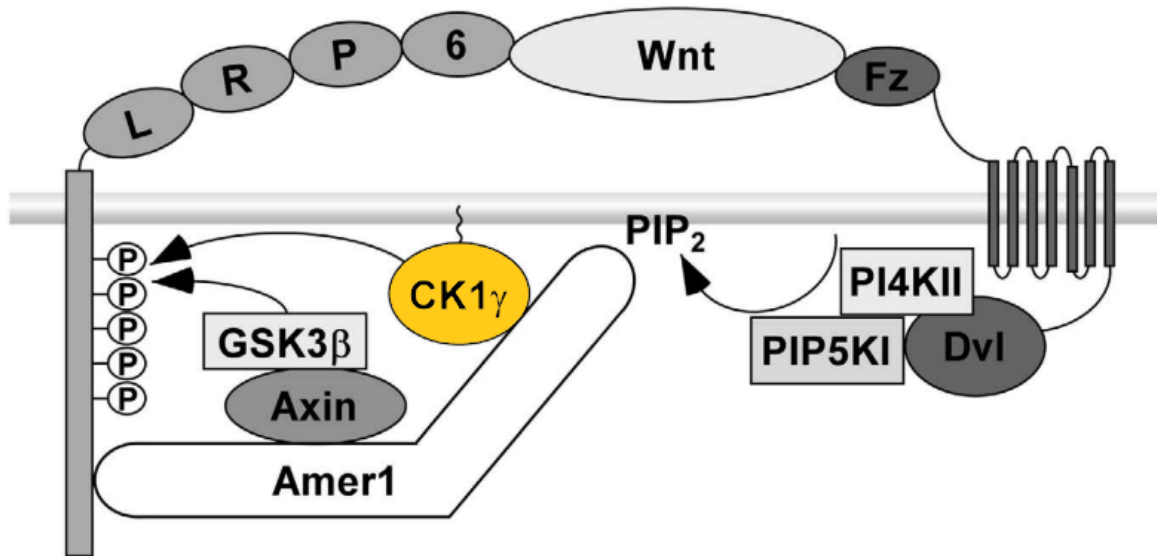


Figure 3: Scheme of initial steps of the Wnt signalling pathway in mammals. The protein Wnt is bound extracellularly to its receptors Frizzled (Fz) and LRP6. Membrane bound CK1gamma phosphorylates LRP6 intracellularly while components of destruction complex Axin and Dvl are attracted to the plasmatic membrane. Taken from (Tanneberger et al., 2011)

1.7 Auxin inducible degron

1.7.1 Principle

Methods for conditional knockdown of proteins were very limited in *C. elegans* until the adaptation of the AID system by Zhang and his team emerged. The AID system is a method designed to quickly, cheaply and effectively induce conditional knock-down. Degradation system was taken from plants but is functional for nematodes as well. The first step is to edit the worm genome by CRISPR/Cas9 editing and add a degron sequence – the auxin inducible degron, AID – at a desired position within the coding sequence of the gene of interest. This degron is designed to be recognized by a plant F-box protein TIR1 only in the presence of the auxin hormone. When auxin is bound to TIR1, the protein is activated and complexed with other E3 ubiquitin ligase complex components. This complex polyubiquitinylates the target degron-labelled protein and sends it for the degradation in the proteasome. As this is happening only in the presence of auxin, the process is reversible (Zhang et al., 2015). Scheme of the degradation process is in Figure 4.

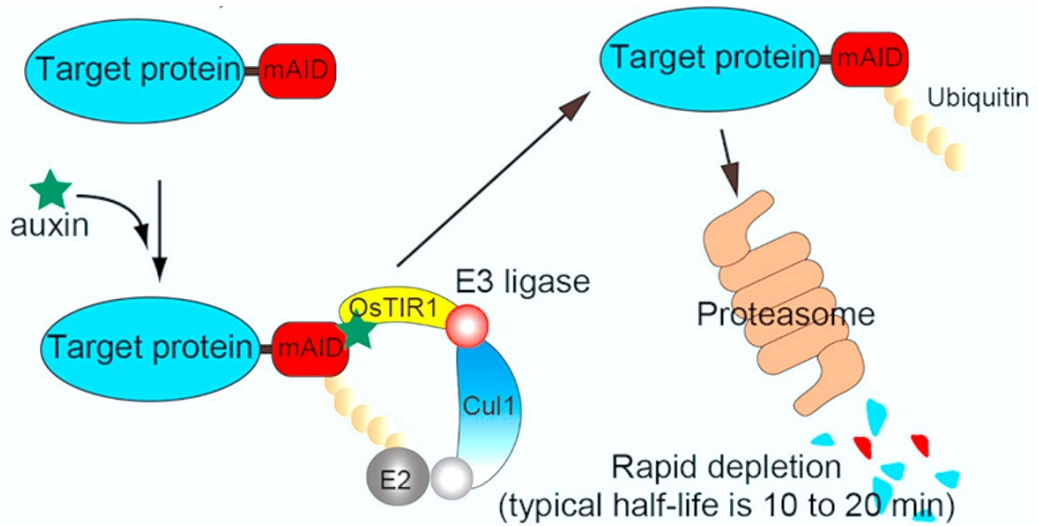


Figure 4: AID system principle. Target protein (cyan oval) was tagged by degron (mAID) sequence using CRISPR/Cas9 approach. This degron is recognized by TIR1 F-box protein (yellow) in the presence of auxin. TIR1 then complexes with other components of E3 ubiquitin ligase and polyubiquitylates target protein to send it for degradation (edited from Natsume et al., 2016).

2 Objective

Casein kinase 1 protein (CK1) family is family of 6 kinases that are important for signal transduction in eukaryotic cells. They play important roles in Wnt/ β -catenin signalling, essential pathway for development and homeostasis of cells. Casein kinases 1 influence Wnt signalling in both positive and negative way. We focused on casein kinase 1 gamma (CK1 γ). In mammalian cells this kinase phosphorylates LRP5/6 coreceptor for Wnt molecule influencing the signalling positively, towards transcription of Wnt regulated genes. As homologous protein to LRP5/6 is missing in *C. elegans* genome, we wanted to know if CSNK-1 can still play a role in Wnt canonical signalling and in which part of it. Our work was divided in four main objectives:

1. To identify if CSNK-1 is contributing on Wnt signalling in *C. elegans* by loss-of-function RNAi experiments.
2. To localize CSNK-1 function to the Wnt producing or receiving cells by overexpression under specific promoters.
3. To determine if CSNK-1 is influencing QL neuroblast polarization.
4. To optimize conditions for auxin inducible degron system, which allows conditional knock-down of target protein in particular time and tissue.

3 Materials and methods

3.1 Strains

3.1.1 Bacterial strains

OP50	<i>E. coli</i> commonly used for <i>C. elegans</i> feeding
DH5 α	<i>E. coli</i> competent strain for plasmid cloning
HT115	<i>E. coli</i> strain used for feeding during RNAi experiments, RNase III deficient

3.1.2 *C. elegans* strains and transgenes

N2 Bristol	wildtype laboratory strain
LGII: <i>muls32</i>	<i>[mec-7p::GFP; lin-15(+)]</i> (Ch'ng <i>et al.</i> , 2003)
	<i>rrf-3</i> <i>pk1426</i>
	<i>mig-5</i> <i>(cp385[mNG-GLO^AID::mig-5]) II</i> (Heppert <i>et al.</i> , 2018)
LGIII: <i>vps-29</i>	<i>tm1320</i>
	<i>mtm-6</i> <i>ok330</i>
LGV: <i>mtm-9</i>	<i>ar479</i>
	<i>muls35</i> <i>[mec-7p::GFP; lin-15(+)]</i> (Ch'ng <i>et al.</i> , 2003)
	<i>hels63</i> <i>[wrt-2p::GFP::PH + wrt-2p::GFP::H2B + lin-48p::mCherry]</i> (Wildwater <i>et al.</i> , 2011)

Mutant strains were obtained from Caenorhabditis Genetics Center, University of Minnesota, USA.

3.2 Cultivation media

3.2.1 Bacteria cultivation media

Lysogeny broth (LB) medium was used for bacteria growth with composition per 1 litre:

10 g	tryptone
5 g	yeast extract

10 g NaCl

For LB plates 15 g of agar was added. Then the water to a total volume of 1 litre was added and the solution was autoclaved. Optionally, antibiotics were added for bacteria selection in RNAi experiments.

3.2.2 *C. elegans* cultivation

Strains were maintained on NGM (nematode growth medium) agar plates which contain the following ingredients per 1 litre:

3 g NaCl

2.5 g Tryptone

20 g Agar

972 ml H₂O

The medium was autoclaved at 120°C. After cooling to 55°C, the following solutions were added:

1 ml Cholesterol (5 mg/ml in EtOH)

1 ml MgSO₄ 1M

1 ml CaCl₂ 1M

25 ml K-phosphate buffer (pH 6.0)

For 1 litre of K-phosphate buffer was used:

108.3 g KH₂PO₄

35.6 g K₂HPO₄

Distilled water to 1 litre was added and the pH was adjusted to 6.0. The final solution was autoclaved.

Completed NGM was poured onto 60mm plates (8 ml on each) and let solidify overnight at room temperature. Simultaneously OP50 bacteria were inoculated into LB media and cultured overnight at 37°C. The next day bacteria culture was spread onto 60mm NGM plates. After another overnight growth of bacteria at room temperature plates were used for *C. elegans* feeding or stored for later use at 4°C.

M9 buffer was used for collecting worms from the plates. For 1 litre:

7.16 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

3 g KH_2PO_4

5 g NaCl

1 ml MgSO_4 1M

All ingredients were dissolved in H_2O added to the total volume of 1 litre and autoclaved.

3.2.3 *C. elegans* bleaching

Bleaching of *C. elegans* is used for decontamination or synchronization of worms. A plate with a large number of gravid adults was washed out with M9 buffer, the solution was transferred to a fresh 1.5ml tube and the worms were let to settle to the bottom of the tube. Then a bleaching solution was mixed using 100 μl of bleach (Savo – 4.7% NaClO) and 50 μl of 1M KOH . The volume of the M9 buffer with worms in the first tube was adjusted to 350 μl and transferred to the bleach solution. After approx. 4 minutes, when worms started to dissolve and break up, the tube was centrifuged at $4,000\times g$ for 30 seconds and supernatant was discarded. The final pellet was washed three times with M9 buffer to get rid of the bleach residues. After the last wash, the embryonal pellet was left in about 50 μl of M9 buffer and pipetted onto a fresh NGM plate. For a better effect of decontamination, small larvae were transferred to a new plate the next day.

3.3 RNAi experiments

3.3.1 RNAi mechanism

RNA interference (RNAi) was first uncovered in *C. elegans* using antisense RNA to disrupt myofilament proteins expression (Fire *et al.*, 1991). Later it was shown that double-stranded RNA (dsRNA) is much more efficient in gene silencing (Fire *et al.*, 1998) and this process is in evolution conserved and useful for research (Hannon, 2002). The function of RNAi in organisms should be to protect the organism from viruses, transposons and non-functional mRNA (Tijsterman *et al.*, 2002).

RNAi is a convenient method of how to decrease or disrupt gene expression in *C. elegans*. There are four delivery methods for dsRNA into *C. elegans*: injection (Fire *et al.*, 1998), feeding (Timmons *et al.*, 2001), soaking (Tabara *et al.*, 1998) and production from

transgenes (Tavernarakis *et al.*, 2000; Wang and Barr, 2005). Membrane proteins SID-1 and SID-2 then provide distribution of dsRNA from the intestine or extracellular space through the worm body. These proteins were found only in *C. elegans* making them excellent models for RNAi experiments (Winston *et al.*, 2007). This dsRNA is then spliced to shorter single-stranded siRNA by leading to targeted destruction of homologous nascent transcripts and mRNA (Zhuang and Hunter, 2012).

3.3.2 RNAi plates

Plates for RNAi were prepared the same way as the common NGM plates with additional ingredients – antibiotics and IPTG. Ampicillin was added to a final concentration of 50 µg/ml, tetracycline to 12.5 µg/ml and IPTG to 1mM final concentration. The RNAi NGM media was poured to 60mm plates and let solidify overnight at room temperature.

3.3.3 RNAi bacteria

HT115 bacteria strains were taken from the Ahringer lab library (Kamath *et al.*, 2000). Each library clone carries an L4440 plasmid with inserted fragment of genomic DNA from the gene of interest. The genomic fragment is flanked by T7 RNA polymerase promoters on both ends to produce dsRNA. Expression of T7 RNA polymerase is inducible by IPTG.

Selected bacterial library clones were inoculated into LB media with antibiotics and incubated at 37°C overnight. Then approximately 50 µl of the liquid culture was spread onto each RNAi plate and let grow overnight to produce dsRNA.

3.3.4 RNAi experiment

To monitor the effect of RNAi, approx. 10 worms in the L4 larval stadium were picked and placed onto a 60mm RNAi plate. One plate was prepared for the negative control, one for positive control and one for *csnk-1*. Plates were then incubated for 4 days at 20°C to see the full effect of RNAi in the progeny. To see how samples for observation and counting under the microscope were prepared, please refer to chapter 3.5.1.

3.4 *csnk-1* cloning

3.4.1 Isolation of RNA

N2 worms were collected from plates with M9 buffer to 1.5ml tubes. They were washed three times with M9 buffer. After the last wash, the pellet was resuspended in 30 µl and 300 µl of Trizol reagent (Thermo-Fisher) was added. The tube was frozen in liquid

nitrogen, thawed and then incubated at 65°C for 15 minutes with occasional vortexing. Next step was to add 60 µl of chloroform, vortex intensively and let rest for 3 minutes at room temperature. Then the solution was centrifuged for 15 minutes at 14,000×g. The upper fraction was collected to a new tube, mixed in 5:4 ratio with isopropanol and left at room temperature for 10 minutes. Then the sample was centrifuged again for 10 minutes at 14,000×g. The supernatant was discarded, and the pellet was washed in 500 µl 75% EtOH. The supernatant was discarded again, and the pellet was dried and then resuspended in DEPC H₂O.

3.4.2 cDNA synthesis

For cDNA synthesis, *SuperScript III First-Strand Synthesis System for RT-PCR* (Invitrogen) was used. To a 0.5ml tube, the following components were added:

5 µg	RNA
1 µl	50µM oligo(dT) primers
1 µl	10mM dNTPs

After DEPC H₂O to 10 µl was added, the mixture was incubated at 65 °C for 5 minutes and placed on ice for 1 minute. Then the cDNA Synthesis Mix was prepared in the indicated order:

2 µl	10× RT buffer
4 µl	25mM MgCl ₂
2 µl	0.1M DTT
1 µl	RNaseOUT
1 µl	SuperScript III RT

Both solutions were mixed and collected by brief centrifugation. Incubation at 50°C for 50 minutes was carried out. The reaction was terminated at 85°C for 5 minutes and chilled on ice. The next step was to collect the reaction by centrifugation, add 1 µl of RNase H and incubate for 20 minutes at 37°C. The sample was then stored at -20°C.

3.4.3 pJet cloning and ligation

As a vector for bacterial transformation pJet1.2 from ThermoFisher was used shown in Figure 5. Cloning was performed according to the cloning protocol from CloneJET PCR cloning kit. Following ingredients were mixed on ice:

10 μ l	2 \times reaction buffer
1 μ l	cDNA
1 μ l	pJet vector (50 ng/ μ l)
7 μ l	nuclease-free water
1 μ l	T4 DNA ligase

The mixture was vortexed briefly and centrifuged for 3-5 seconds. Then the incubation in room temperature for 5 minutes was carried out. This solution was directly used for bacterial transformation. Left-overs were stored at -20°C.

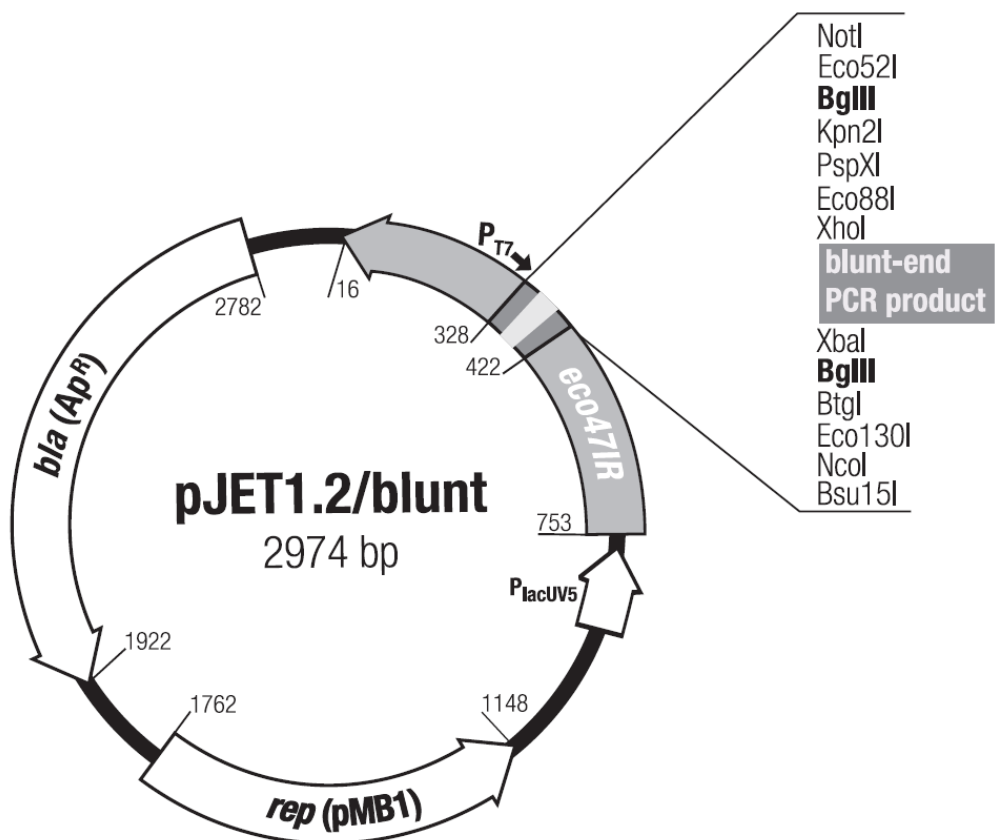


Figure 5: Map of pJET1.2 plasmid from ThermoFisher

3.4.4 Bacterial transformation

DH5 α competent bacteria for DNA plasmids transformation were taken from -80°C and put on ice to slowly thaw. Then 5 μ l of the ligation mixture was gently mixed with the bacteria and let rest on ice for 30 minutes. The next step was to put the bacteria at 42°C to induce heat shock for 80 seconds and then let them cool again on ice for 5 minutes. After that, 750 μ l of LB media was added and the mixture was incubated at 37°C for 45 minutes to start growth. Produced bacterial culture was concentrated by centrifugation and 100 μ l was spread to 120mm LB agar plate with appropriate selective antibiotics, where only successfully transformed bacteria were able to grow. The plate was left overnight at 37°C. The next day the grown colonies were picked up.

3.4.5 Gibson assembly

The Gibson assembly cloning method (Gibson *et al.*, 2009) allows simultaneously connecting more than two fragments of DNA into one plasmid. The crucial part of this method is to design primers with overlapping ends to the next fragment. On every border between two fragments, there are two primers – one with standard length (about 20 nt) and one with overlapping end to the next fragment (about 30 nt). After the PCR reaction, due to these overlapping sequences, it is possible to connect the DNA fragments to one big plasmid by ligation. Ligation mixture (G-mix) contains all extended fragments mixed with nucleotides, DNA endonuclease, DNA polymerase and DNA ligase.

We intended to create plasmids for overexpression of CSNK-1. We wanted to connect three fragments – promoter to reach tissue-specific expression in cells producing Wnt (*egl-20* promoter) and cells receiving Wnt signal (*egl-17* promoter),

For one PCR reaction was used:

10 μ l	5 \times HF buffer
1 μ l	dNTPs
2.5 μ l	forward primer
2.5 μ l	reverse primer
33 μ l	ddH ₂ O
0.5 μ l	DNA sample

0.5 μ l Phusion DNA polymerase

PCR cyclers was set as follows:

98°C 2 min. initial denaturation

98°C 30 s denaturation

60°C 30 s primer annealing

72°C 1 min/kb polymerase elongation

Steps 2 to 4 of the cycle were repeated 35 \times

Used primers:

egl17GFP-R

ACAACCTCCAGTGAAAAGTTCTTCTCCTTTACTCATAGCTCACATTTTCGGGCACC

egl20p-GFP-R

ACAACCTCCAGTGAAAAGTTCTTCTCCTTTACTCATTATTTCTGAAATTGAGATGTTTTAG

csnk1-unc54UTR-R

ATGGCGATCTGATGACAGCGGCCGATGCGGAGCTCCTATTTTTGTGTAGCTGGGGTCCG

GFPcsnk1-F CTGGGATTACACATGGCATGGATGAACTATACAAAATGACGAACACACGCGGGA

unc54-3UTR-F GAGCTCCGCATCGGCCGC

GFPcel-F ATGAGTAAAGGAGAAGAAGACTTTTC

GFPcel-R TTTGTATAGTTCATCCATGCCA

The first two primers were specific for promoters, first four primers are extended.

In our case, we needed to connect pPD95.81 vector with the desired promoter, GFP tag and csnk-1 cDNA in one construct showed in Figure 6. Therefore 3 μ l of vector DNA, 1 μ l of GFP DNA and 1 μ l of csnk-1 DNA was mixed to G-mix and put for 60 mins at 50°C.

G-mix was prepared from the following:

320 μ l 5 \times ISO buffer

0.64 μ l 10 U/ μ l T5 exonuclease

20 μ l 2 U/ μ l Phusion polymerase

160 μ l 40 U/ μ l Taq ligase

The water was added to 1.2 ml and the mixture was aliquoted (15 μ l aliquots) and stored at -20 °C.

5 \times ISO buffer was prepared from the following per 1 ml:

0.5 ml 1 M Tris-HCl pH 7.5

25 μ l 2 M MgCl₂

10 μ l of each 100 mM dNTP

50 μ l 1 M DTT

0.25 g PEG-8000

50 μ l 100 mM NAD

Distilled water to 1 ml was added and the buffer was aliquoted (320 μ l aliquots) and stored at -20 °C.

3.4.6 Injection of plasmids

Created vectors were injected to *vps-29; muls32* worms using the gonadal microinjection technique (Mello *et al.*, 1991) together with a red fluorescent marker *myo-2p::tdTomato* to facilitate recognition of successfully injected worms. After injection, the worms were split one by one on individual plates. From their progeny, worms with red fluorescence in the pharynx were selected as the F1 generation. If some worms from the F2 generation also had red pharynxes, then a stable line was acquired. We have obtained 3 stable lines with the *egl-20* promoter constructs and 2 stable lines with the *egl-17* promoter construct.

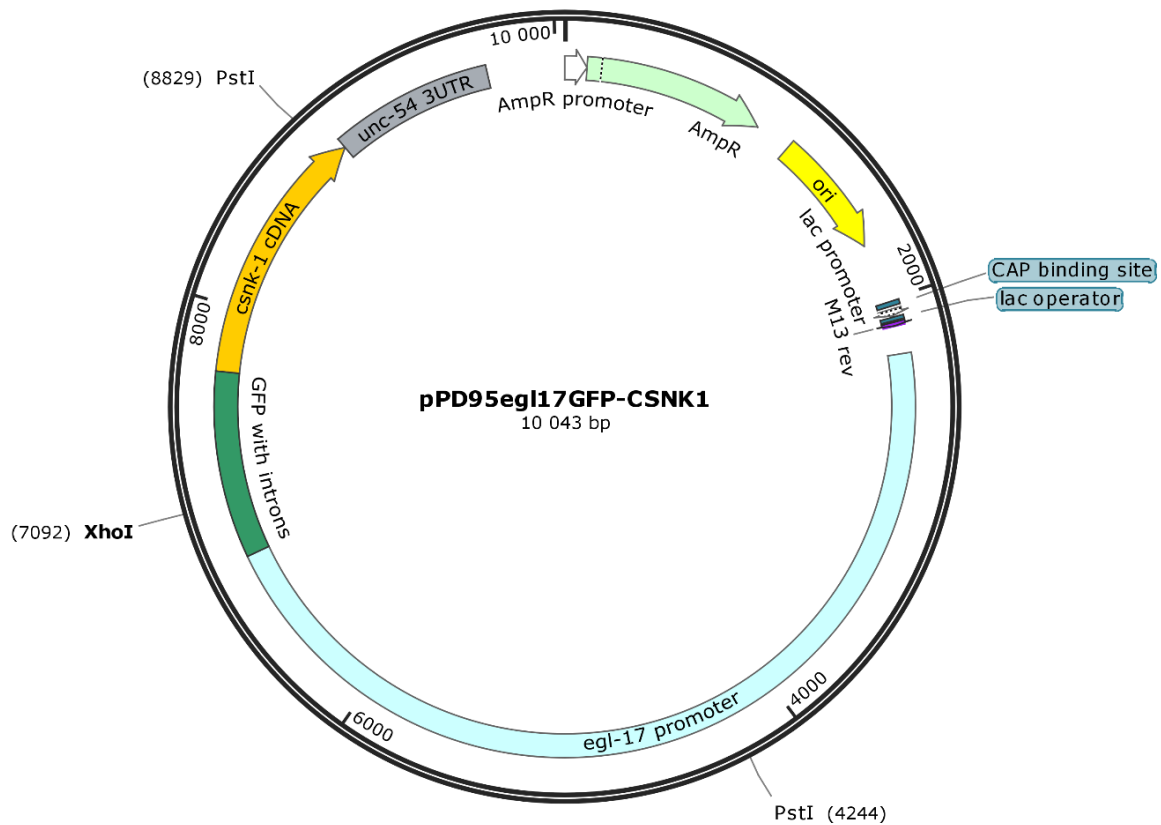


Figure 6: Map of plasmid for overexpression. The plasmid was created by Gibson assembly approach from 3 fragments. It contains an *egl-17* promoter (light blue), GFP (green), *csnk-1* cDNA (orange), *unc-54* 3'UTR (gray) and ampicillin resistance for selection. The map was created in SnapGene.

3.5 Early QL migration experiments

To synchronize and obtain eggs, regular bleaching method was used starting with one full plate of gravid adults as previously described in chapter 3.2.3. Eggs were left to hatch for one hour in room temperature and newly hatched larvae were then transferred to a fresh plate and let developing for one, two or three hours depending on the QL migration stadia we wanted to observe. Worms were prepared for microscopy in the way described in chapter 3.7.2.

Due to long time observation under the microscope, worms tended to dry quickly. For this reason, slides were moistened by distilled water every few minutes to maintain worms in a good condition for observing.

3.6 AID system

3.6.1 Auxin plates

To induce protein depletion, we used standard NGM plates with 1mM auxin. As auxin, we used indole-3-acetic acid from Alfa Aesar (catalogue no. AAA1055606). Auxin solution was made from loose powder auxin dissolved in 98% EtOH to 400 mM concentration and stored for a maximum one month at 4°C. Concentrated auxin was added to agar after cooling to 55°C together with cholesterol and salts to a final concentration of 1 mM.

We found out that OP50 bacteria do not grow well on auxin plates even at low auxin concentrations and scarcity of food was then slowing down *C. elegans* life cycle. Therefore, bacteria were usually concentrated before spreading onto the plates. Auxin plates were used within one month.

3.7 Microscopy and image editing

3.7.1 Worm preparation for microscopy

At first, the worms were flushed out with M9 buffer from plates to 1.5ml tube. Meanwhile, 150 µl of warmed 3% agarose with 10mM NaN₃ was pipetted on a glass slide and immediately covered with another slide to create a thin layer of agarose. After a few seconds, the upper cover slide was removed and 5 µl of M9 with worms was pipetted onto the agarose layer and covered with a coverslip. The NaN₃ in the agarose ensures immobilization of the worms and provides the opportunity to observe and count them under the microscope.

3.7.2 Microscopy

For microscopy, Leica DM6 upright fluorescence microscope was used. Worms were observed mainly using differential interference contrast and fluorescence microscopy using the blue light spectrum for GFP excitation.

3.7.3 Image editing

For image editing and montage composition, free software FIJI (Schindelin *et al.*, 2012) and Inkscape were used.

3.8 Others

3.8.1 Electrophoresis in agarose gel

TBE (Tris, borate, EDTA) buffer was used for molecular weight checks, for DNA fragments isolations TAE (Tris, acetic acid, EDTA) buffer was used. The concentration of agarose was always 1%. For visualisation of DNA under UV light ethidium bromide was used to the final concentration 5 µl/ml, for size determination 1kb DNA ladde.

3.8.2 Plasmid DNA isolation

For isolation of DNA (plasmids) from bacteria, High-Speed Plasmid Mini Kit by Geneaid was used. The protocol is available from <http://www.geneaid.com/products/plasmid-dna-purification/plasmid-kit-miniprep>.

4 Results

4.1 RNAi

Preliminary experiments performed in the laboratory indicated that CSNK-1/CK1 γ function is required for Wnt-dependent Q cell migration (M. Macůrková, unpublished). We decided to test this observation further. Due to the lethality of the *csnk-1* knockout, as we can see in tm1762 allele (*C. elegans* Deletion Mutant Consortium, 2012; Flynn and McNally, 2017), we were not able to study CSNK-1 activity using a complete loss-of-function approach. We, therefore, decided to lower the expression of this kinase by RNAi feeding technique to achieve the reduction of function.

The initial experiments were made in an otherwise wild-type strain, which carries an integrated *mec7p::GFP* transgene *mulS32* (Ch'ng *et al.*, 2003). This transgene allows easy visualisation of QL neuroblast progeny. Worms in L4 larva were fed on NGM plates with RNAi bacteria and their progeny was observed under the microscope for QL phenotype. Feeding with bacteria containing empty L4440 vector or vector with *snx-3* genomic fragment was used as a negative and positive control, respectively. The assay was repeated three times with 100 animals counted each time. Unfortunately, no effect on the phenotype was observed.

Another strain *rrf-3;mulS32* sensitive for RNAi, due to a loss of RRF-3 RNA-directed RNA polymerase (Simmer *et al.*, 2002), was used for the same assays. No effect was observed in QL neuroblast migration as well as Lehner and his team found out previously (Lehner *et al.*, 2006).

As a result, we decided to try *csnk-1* RNAi in a mutant background to see if the exhibition of QL phenotype will be higher if we interrupt Wnt signalling on more levels. Firstly, we started with *vps-29* mutant, which is retromer complex component playing role in Wnt secretion. Secondly, we tested *mtm-6* and *mtm-9* (myotubularin related protein) mutant strains, which are also part of the Wnt secretion. Assays were done in the same way as the previous ones, repeated 3 times on the set of 100 animals with negative and positive control.

All of these mutant strains had a significant increase in QL phenotype (Fig. 7), which can mean that CSNK-1 influences WNT secretion, WNT reception or early QL neuroblast migration in the L1 larval stadium.

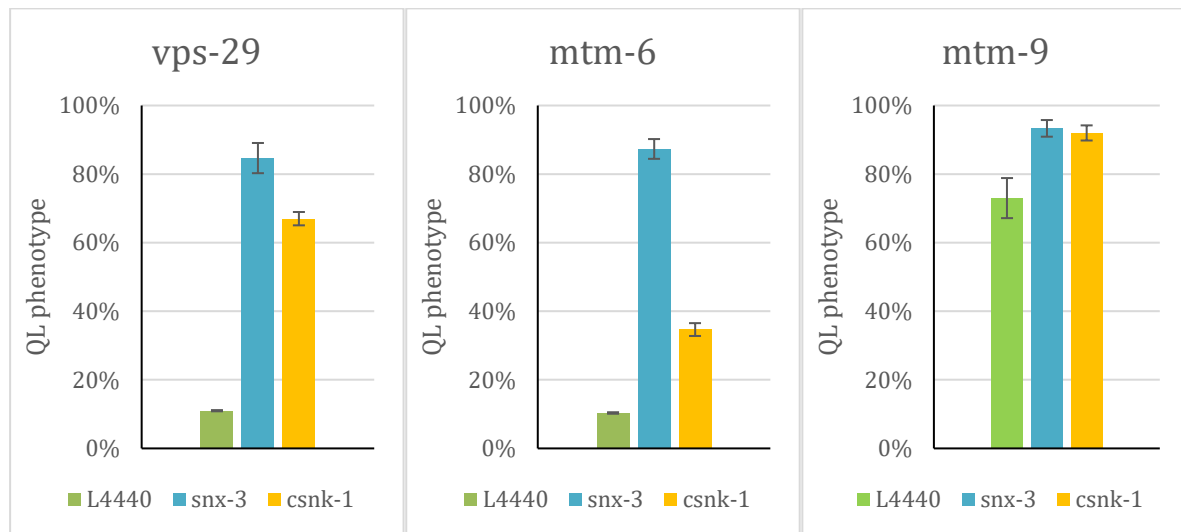


Figure 7: QL phenotype after *csnk-1* RNAi in different genetic backgrounds. These 3 graphs show a significant increase in QL phenotype in *csnk-1* RNAi animals (yellow column) compared to negative control empty L4440 vector (green column). In all experiments, there was *snx-3* RNAi (blue column) as a positive control. The experiment was taken in different genetic backgrounds – *vps-29*, *mtm-6* and *mtm-9*. Bars represent mean values of three independent experiments with 100 animals in each experiment, error bars represent standard deviations. Data were analysed with unpaired Student *t*-test ($p < 0,05$).

4.2 Overexpression

We found out from previous experiments the possible influence of CSNK-1 on Wnt signalling by knocking-down the expression of it. Our next question was, what will happen if we overexpress CSNK-1 under specific promoters. We made two plasmids with *csnk-1* under different promoters – *egl-17* promoter, for cells receiving Wnt signal, which are QL neuroblast in L1 and vulval cells precursors at L4 larval stadium, and *egl-20* promoter, for cells producing Wnt signal around the rectum (Whangbo and Kenyon, 1999). The plasmids were injected to *vps-29; muls32* mutant strain to enhance the effect of *csnk-1* overexpression similarly to the previous down-regulation experiments.

From F1 and F2 generation of injected animals were selected transgenic animals based on *myo-2::mCherry* marker. We gained two transgenic strains with local overexpression for each plasmid. Then we inspected these animals under the microscope for expression

of *GFP::csnk-1*, results are shown in Figure 8. We were observing precursors of vulval cells at L4 and QL neuroblast at L1 stadia in worms with *egl-17* promoter and cells close to rectum at L1 stadia in *egl-20* transgene worms. Unfortunately, we did not see any GFP signal in QL cell of *egl-17* worms.

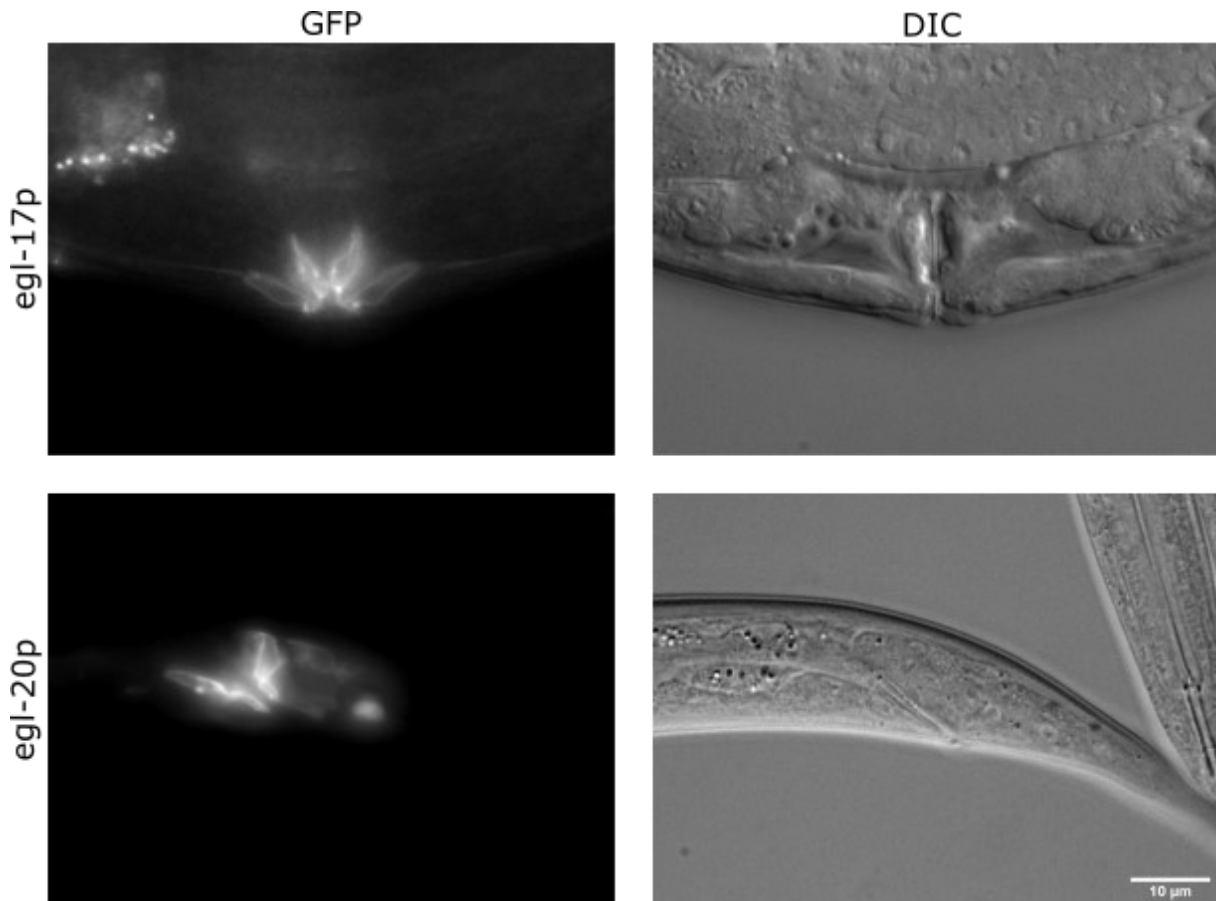


Figure 8: Local expression of the GFP::csnk-1. The expression of GFP::csnk-1 under egl-17 promoter (first row) in vulva at L4 larval stadium and expression of GFP::csnk-1 under egl-20 promoter (second row) in cells around the rectum. Worms are oriented anteriorly to the left.

We were also observing these animals for QL phenotype and found no relevant difference between transgenic and non-transgenic animals on the same plate. There were 100 worms counted in each category and scoring was repeated at least three times.

4.3 Early QL polarization

As we realize that CSNK-1 influences QL migration, we wanted to see in which part of the migration is it happening. In wild-type worm, this migration is under control of two different pathways. In the first larval stadium, QL neuroblast polarizes and migrates posteriorly over the V5 seam cell. Polarization and this short-range migration are not dependent on Wnt signalling, but on UNC-40/DCC, DPY-19/DPY-19L1, PTP-3/PTPRD and

MIG-21 membrane proteins (Honigberg and Kenyon, 2000; Middelkoop *et al.*, 2012). The second pathway comes after the first QL division and is tightly coupled with EGL-20/Wnt signalling and *mab-5* expression.

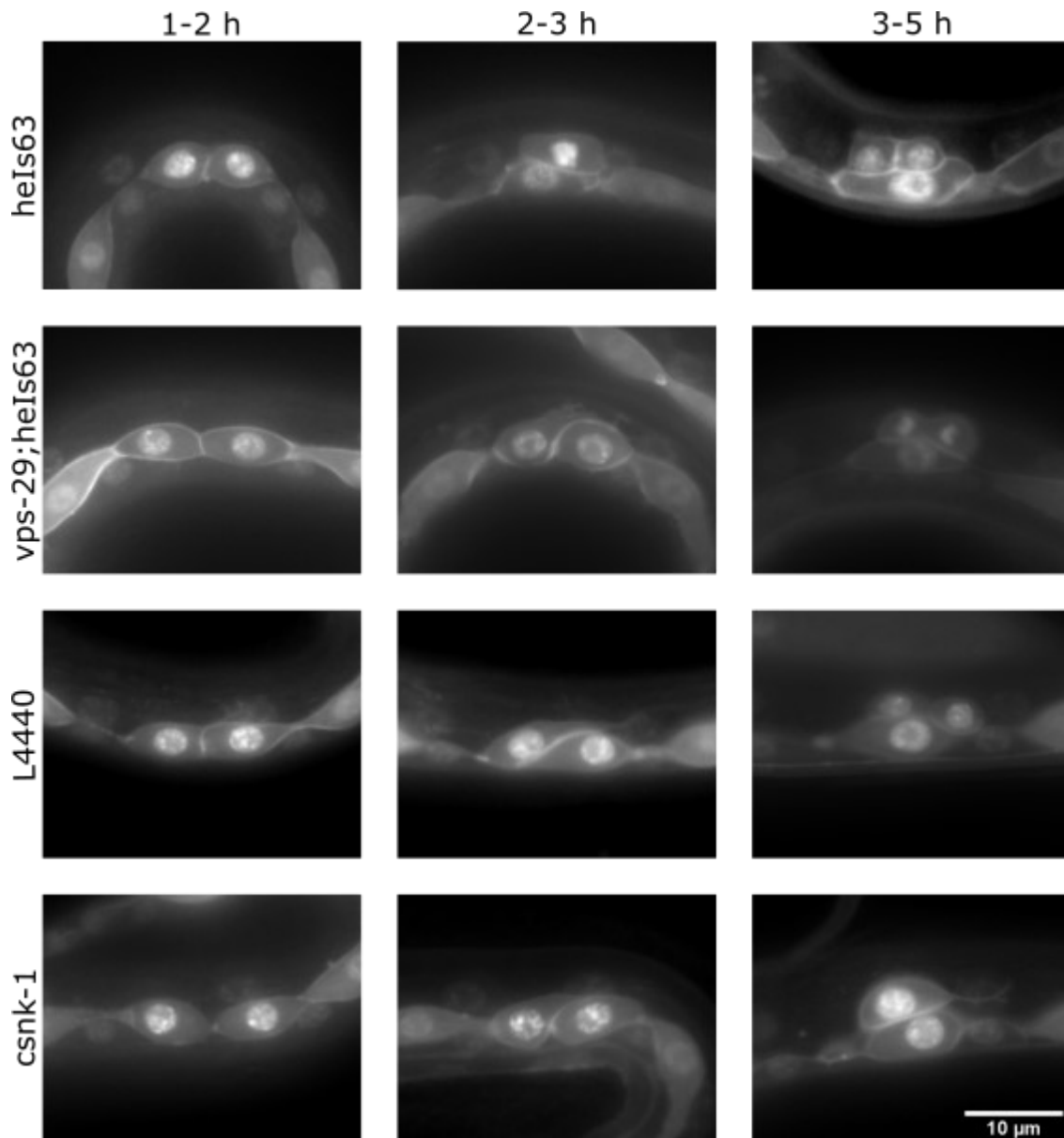


Figure 9: QL neuroblast early migration. QL neuroblast polarization and migration over V5 seam cell toward posterior and subsequent sensitisation to WNT and cell division in time. Strain heIs63 and vsp-29;heIs63 as a negative control in upper rows, and lower rows L4440 RNAi in vps-29;heIs63 as a negative control for RNAi and csnk-1 RNAi in the same strain vps-29;heIs63.

It was previously shown that CSNK-1 can play a regulatory role in polarization and asymmetric division of *C. elegans* embryo. Loss of *csnk-1* by RNAi in embryos was causing massive spindle and pronuclear movement occasionally leading to symmetric divisions instead of asymmetric (Panbianco *et al.*, 2008). Based on these findings we decided to

observe QL neuroblast in the earliest stage to see if CSNK-1 is changing the direction of migration in this initial polarization phase or later in Wnt signalling.

Worms were bleached to gain eggs and let hatched for one hour. Hatched larvae were transferred on the fresh plate and were developing for another hour, 2 hours or 3 hours depending on stadia of QL migration we wanted to see. Then the worms were prepared for microscopy as described in chapter [3.7.2](#). We utilized a strain carrying an integrated transgene *hels63*. This transgene carries PH domain and histone H2B both tagged with GFP to visualise the plasma membrane and the nucleus. The same transgene was also crossed to *vps-29* mutant background. The initial QL migration was first observed in these two strains to make sure that the transgenic modifications are not making any influence on QL polarization and migration direction. Then the *vps-29;hels63* was treated with *L4440* RNAi as a negative control or RNAi with *csnk-1* RNAi. As shown in Figure 9, QL neuroblasts in all experiments were behaving the same. They were polarizing and migrating towards posterior over V5 seam cell during the first 3 hours and dividing into two cells shortly after that. The only difference we observed in *csnk-1* RNAi was slightly lagged migration and division of QL for about an hour behind the other strains.

4.4 AID system

4.4.1 Auxin experiments

For future studies of CSNK-1 function, it would be beneficial to introduce the auxin-inducible degron system to be able to regulate expression levels precisely and tissue-specifically. But before using the AID method for CSNK-1, it was necessary to optimize the duration of auxin induction, an optimal larval stadium for induction and tissue specificity, using a protein with known function and knock-down phenotype. For this purpose, we used *mig-5*, which is *C. elegans* homolog for mammalian Dvl protein. We obtained a CRISPR/Cas9-engineered strain carrying *mig-5* tagged with *mNeonGreen* and the AID (*cp385[mNG-GLO^AID::mig-5]*) (Heppert *et al.*, 2018). If MIG-5 is degraded in Wnt-receiving cells, the treated animals should exhibit QL phenotype. This phenotype was observable due to *mul35* transgene crossed into the AID-edited one. The *mul35* strain has the same properties as *mul32*: visualisation of some neurons including one QL descendant, PVM, but the modification lies on a different chromosome.

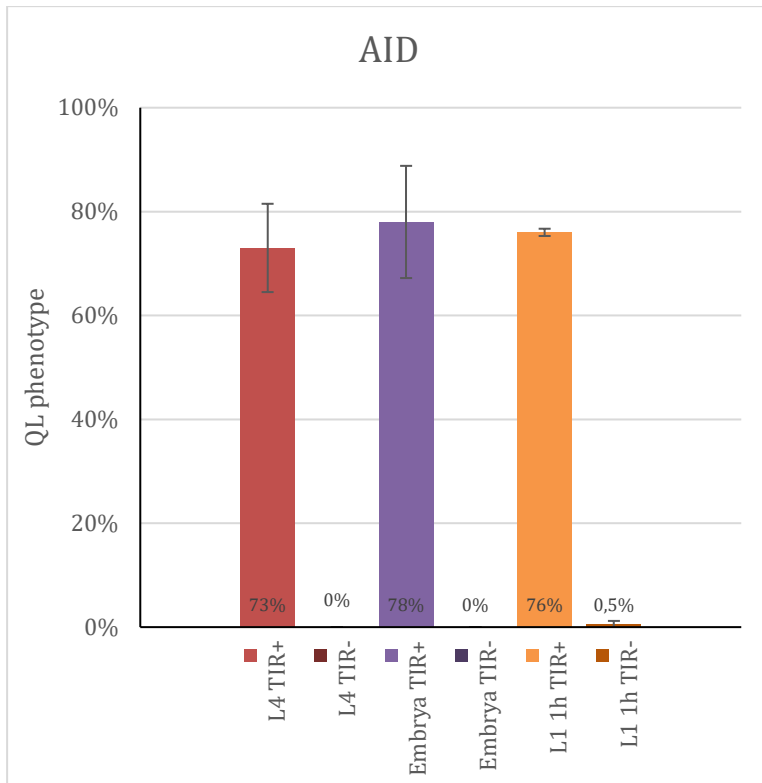
Degradation of MIG-5 protein was at first induced by TIR1 expressed from a ubiquitously active *eft-3* promoter. Results showed quite robust induction of the degradation (Figure 10). The first tested stadia were L4 placed on NGM plate with 1mM auxin. We were observing their progeny at L4 stadia looking for QL phenotype and loss of green fluorescence of MIG-5 protein indicating its degradation.

To shorten the time for MIG-5 protein depletion, we bleached gravid worms with KOH and bleach solution to dissolve worms' bodies and obtain embryos. These embryos were washed and laid on auxin NGM plate, let hatch and develop till the L4 stadia for the observation under the microscope.

The last experiment with *eft-3* promoter was on 1-2h L1 larvae. The abundantly grown plate was washed multiple times with M9 buffer to get rid of larvae and adults, sticky eggshells prevented embryos to wash away from the plates. Embryos on the plate were let hatched for an hour. Newly hatched L1 larvae were transferred to the auxin plate and let grow to the L4 stadia for microscopy. Unfortunately, with this approach, we got a very low number of worms and even lower number of transgenic ones. For this reason, we decided to gain embryos in the same manner as the previous experiment, by bleaching. Bleached embryos were laid on a regular NGM plate to hatch. After an hour, hatched larvae were transferred to the auxin NGM plate and let developed to the L4 state and observed.

In all experiments with the *eft-3* promoter, we saw more than 70% occurrence of the QL phenotype leading to the assumption that MIG-5 was successfully depleted with the visual confirmation by the considerable lowered green signal. However, we were not able to reach almost complete depletion as previous researchers were (Zhang *et al.*, 2015).

Then we expressed the TIR1 protein under *egl-17* and *egl-20* promoters for tissue-specific expression in cells sending and receiving Wnt signal. In case of this specific expression, we should see QL phenotype only when expressing TIR1 in the in receiving cells (*egl-17* promoter) as MIG-5/Dvl protein is acting in this part of the canonical Wnt signalling. On the other hand, in worms with TIR expressed under *egl-20* promoter, we should see wild-type QL migration. However, neither *egl-17* nor *egl-20* driven TIR1 showed any effect on QL migration, also the green fluorescence from the *mNeonGreen* tag was the same in comparison to the negative control.



*Figure 10: QL phenotype after MIG-5 degradation at various developmental stages. This graph is showing the percentage of QL phenotype in the strain *eft-3p::TIR1; muls35; mig-5::AID* after transfer to the auxin plates. On the plate were transferred L4 larvae and their progeny was observed for the phenotype (in red) or freshly bleached eggs and they were observed in L4 stadia (in purple) or L1 larvae 0-1hour after hatching and observed in L4. Experiment for all stages was repeated at least three times. In L4 category 300 worms were counted, in embryo category 272 and L1 1h category 116 worms. Strain *muls35::mig-5::AID* without extrachromosomal TIR was used as a negative control.*

5 Discussion

The function of casein kinase gamma 1 in mammals was uncovered in 2005 by Davidson and his team. They found out that membrane-bound kinase CK1 γ can phosphorylate LRP6 receptor on multiple sites to promote Wnt signalling. This phosphorylation is sufficient and necessary at the same time for this signalling (Davidson *et al.*, 2005). LRP5/6 homolog does not seem to be present in *C. elegans* (Korswagen, 2002), yet preliminary observations have suggested that loss of *csnk-1/CK1 γ* expression leads to similar effect as disruption of the Wnt signalling pathway. Our aim was to bring some insight into how CSNK-1 can possibly regulate Wnt signal transduction in *C. elegans*.

5.1 RNAi experiments

The first task was to find the best way how to study the effects of loss of *csnk-1* expression. Previous research showed early larval lethality for non-functional allele *tm1762* (Consortium, 1998). As *csnk-1* expression is indispensable for embryonal development, persistent knock-out of this kinase was therefore not an option. Instead, RNAi by feeding was thus selected as the first method of choice. This method usually results in an incomplete elimination of the transcript and thus could overcome the lethality and let worms grow and hatch quite normally.

First RNAi assays were done in an otherwise wildtype strain carrying the *mec-7::GFP* marker *muls32* (Ch'ng *et al.*, 2003) to visualize QL neuroblast descendant. We did not see any effect in this strain on QL neuroblast migration after RNAi feeding.

Another strain we decided to try for *csnk-1* RNAi was a strain carrying mutation in *rrf-3* gene. This strain is missing a RNA dependent RNA polymerase, which leads in some cases to an enhanced efficiency of RNAi (Simmer *et al.*, 2002). However, this was not the case for *csnk-1* RNAi. No visible phenotype on QL neuroblast was observed in this strain. This result is not so surprising as previous large scale RNAi screens in *rrf-3* mutant background showed several developmental defects and partial embryonic lethality, but no problems with QL descendant migration were reported (Simmer *et al.*, 2003).

Another approach to enhance the RNAi effect was to use mutant strains, where the Wnt pathway activity has already been decreased. For this purpose, we used mutants in genes

influencing the efficiency of Wnt secretion. These genes were: *vps-29*, coding for component of the retromer complex (Coudreuse *et al.*, 2006; Prasad and Clark, 2006); *mtm-6* and *mtm-9*, genes coding for myotubularin related proteins (Silhankova *et al.*, 2010). Strains carrying mutations in these genes display a partially penetrant QL phenotype, but *csnk-1* RNAi raised the frequency of individuals with this phenotype significantly. These data suggest that CSNK-1 may function in Wnt signalling, although they do not give a hint where in the pathway the kinase could play a role.

One of the reasons for the failure in the initial experiments to detect any QL defects could be low efficiency of RNAi by bacteria feeding. Another option would be to deliver dsRNA by injection. If we compare dsRNA delivery by feeding and injection, we can say that feeding is less labour-intensive and yield more affected animals than injection. Conversely, by feeding we cannot control the amount of dsRNA ingested by the worm, so there is high variability between individuals, whereas during an injection we are delivering an exact concentration of dsRNA (Timmons, 2006). This can cause lower frequency and severity of phenotypes in fed worms in comparison to injected ones as demonstrated in previous work (Timmons and Fire, 1998).

On the other hand, we can also find experiments showing, that efficiency of RNAi by feeding is almost the same as RNAi by injection. This RNAi screen was done by Kamath and his colleagues, where they were looking for perfect optimisation of this method. They even suggested this method as a better option for post-embryonic phenotypes (Kamath *et al.*, 2000).

The general problem with RNAi lies in the sensitivity of this method to different external influences. These influences could be microbial contamination on the plates, growth temperature or developmental stadia at the time of feeding. These condition can make reproducibility of the previously seen phenotypes difficult (Hull and Timmons, 2004; Timmons, 2006). On top of that, it was found that results between the laboratories and even in the same laboratory for one gene could vary between 10-30%, when using the same methods (Simmer *et al.*, 2003).

Unlike other laboratories previously, we did not see larval lethality of about 45 % in *csnk-1* RNAi by feeding (Panbianco *et al.*, 2008; Flynn and McNally, 2017). In our experiments, we were only able to detect low level of lethality and slower development. The most likely

explanation of these results is probably low efficiency of the RNAi in the conditions that we had, therefore *csnk-1* downregulation was presumably incomplete.

Apart from the technical limitations of the experimental setup there are also biological reasons for not seeing QL migration defect by *csnk-1* RNAi in wild type background. One of these reasons could be compensation of CSNK-1 knockdown by a different kinase as the casein kinase 1 protein family is known for high substrate promiscuity. For example, mammalian CK1 δ and ϵ are very similar in structure and have overlapping functions (Schitteck and Sinnberg, 2014). There are speculations whether CK1 γ /Gish is the only CK1 kinase phosphorylating LRP5/6 or CK1 α/ϵ can at least partially substitute this function (Davidson *et al.*, 2005; Zeng *et al.*, 2005; Zhang *et al.*, 2006) The genome of *C. elegans* encodes 438 kinases and 84 of them belong to the CK1 family, while in humans the CK1 family contains only 6 members (Cheong and Virshup, 2011; Zaru *et al.*, 2017). It is thus quite probable that redundancies will exist among the *C. elegans* CK1 proteins.

Another reason could be that under normal conditions CSNK-1 activity is not necessary for Wnt signaling and the amount of Wnt molecule is sufficient to activate Wnt responsive genes without CSNK-1 cooperation. But if the amount of Wnt molecule is lower as in the case of retromer or myotubularin mutants (Coudreuse *et al.*, 2006; Yang *et al.*, 2008; Silhankova *et al.*, 2010), the activity of CSNK-1 becomes necessary to transduce the signal successfully. If we decrease *csnk-1* expression under these conditions, the phenotype would manifest itself and QL.d migration would be inverted.

Taken together, our RNAi analysis revealed that CSNK-1 activity is required for Wnt-directed QL migration at least in a situation where the amount of secreted Wnt is less than optimal. Although RNAi cannot be a full substitution for gene knock-out, results are still relevant for protein function analysis and can mimic loss of target protein (Hull and Timmons, 2004).

5.2 Overexpression

The loss-of-function experiments have a clear logical justification: when the protein is partially or fully depleted or non-functional, mutant phenotype will manifest. However, knock-out or RNAi applied to the whole body does not give any information about the putative site of action of the studied protein. One of the approaches to uncover the specific cell or tissue where the protein is required is site-specific overexpression. As CSNK-1 is a

kinase, one could presume that increased activity of the enzyme at its site of action could also affect the Wnt pathway and would result in an observable phenotype.

We used two promoters associated with canonical Wnt signalling in *C. elegans* to specifically overexpress CSNK-1: *egl-17* promoter and *egl-20* promoter. *Egl-17* encodes an ortholog of human FGF and is expressed early in QL neuroblast and later at L4 stadia in vulval precursors and other cells (Burdine *et al.*, 1998; Branda and Stern, 2000). In our experiments, expression from *egl-17* promoter was thus used to manipulate cells receiving Wnt signal. *Egl-20* encodes promoter one of *C. elegans* Wnts and is expressed in cells around rectum (P9/10, K, F, U, B, mu anal) in L1 stage, later in vulval cells, somatic nervous system and body wall muscle cell (Whangbo and Kenyon, 1999; Harterink *et al.*, 2011). In our experiments *egl-20* promoter was used to manipulate cells transmitting Wnt signal.

Expression of CSNK-1 was detected in all transgenic lines we made. We were able to see signal in vulval precursors cells in worms with *egl-17* promoter construct at L4 stadia and in cells around rectum in worms with the *egl-20* promoter construct during L1 stage. The only problem was that when using *egl-17* promoter there was no visible signal in the QL neuroblast in L1 in neither of the strains we made. This could be explained either by the fact that *egl-17::GFP::csnk-1* is expressed in QL neuroblast, but the signal is very weak, so we cannot detect it, or that our construct is missing some promoter element required for expression in the Q lineage. Anyway, in neither strain we generated there was any difference in QL migration between the individuals carrying the transgene and their siblings without the transgene.

The level of overexpression varies with the diverse methods and target proteins; thus it is not easy to predict results of overexpression experiments (Moriya, 2015). Possible effect of overexpressed CSNK-1 under either of the promoters could be overactivation of Wnt signalling in QL and ectopic Wnt signalling activation in QR neuroblast and subsequent posterior QL-like migration of the QR neuroblast. But we did not see any migration defect in our experiments. This observation could be explained in multiple ways. First, CSNK-1 has a function in Wnt secreting cells, but the increased activity of CSNK-1 does not influence the amount of secreted Wnt and therefore will not influence the phenotype. Second, CSNK-1 could have a role in Wnt receiving cell, QL in our case, but again the increased activity does not influence the signalling outcome. In prior work in

Drosophila, overexpression of Gish/CK-1 γ had no effect on Wg/Wnt signalling compared to loss-of-function experiment (Zhang *et al.*, 2006) and in human HEK293T cells, overexpressed CK1 γ /CSNK-1 was still phosphorylating T1479 of LRP5/6 (Davidson *et al.*, 2005) suggesting that no visible phenotype during CSNK-1 overexpression in *C. elegans* could be a natural outcome of the experiment. As we did not see CSNK-1 expression in the QL in our *egl-17* promoter transgene, the last possibility is that our *egl-17* promoter transgene is not functional in the QL neuroblast. Therefore, no conclusive answer can be drawn from these experiments.

5.3 Early QL migration

From the RNAi experiments we found out, that CSNK-1 influences QL neuroblast migration. This migration is driven by two pathways before the first division: initial polarization signalling and Wnt/ β -catenin pathway. Initial polarization precedes the moment when the QL neuroblast reacts to the Wnt signal and requires UNC-40/DCC, DPY-19/DPY-19L1, PTP-3/PTPRD and MIG-21 proteins, however the exact mechanism of this signalling is not clear (Honigberg and Kenyon, 2000; Middelkoop *et al.*, 2012; Sundararajan and Lundquist, 2012). Some of these proteins are bound to the membrane similarly to CSNK-1. On top of that CSNK-1 was previously shown to participate on cell polarization in collaboration with the PAR proteins (Panbianco *et al.*, 2008). We could not therefore exclude, that rather than acting within the Wnt pathway, CSNK-1 might act in the initial polarization step before the Wnt signalling is active.

To find out if CSNK-1 can participate in the initial polarization, we designed experiment using a *hels63* transgene visualizing seam cells and the Q neuroblast (Wildwater *et al.*, 2011). We expressed this transgene in wild type worms and in *vps-29* mutants to weaken the Wnt pathway as we saw effect of CSNK-1 in this background in previous experiments. We were watching direction and timing of the early QL migration and subsequent division.

These experiments were quite time-consuming and needed to be precisely scheduled to give data comparable between different strains. We were dealing with small number of worms on the glass and not all of them were appropriate for QL neuroblast microscopy. Nevertheless, our observations did not reveal any changes in initial QL polarization after *csnk-1* RNAi in either wild type or *vps-29* mutant background. This suggests that CSNK-1

is more likely to act in the Wnt pathway during QL migration and not during the initial polarization. This conclusion can also be supported by the fact that PAR proteins have not been linked to the Q cell polarization, mechanism of CSNK-1 action in polarization thus would not be clear.

5.4 AID system

Experiments with *csnk-1* RNAi and overexpression revealed some limitations of the experimental design and have shown that a technique allowing for precise spatial and temporal manipulation of protein levels would be beneficial. Auxin inducible degron system is a method for local conditional knock-down of the target protein. This system was taken from plants and adapted for other organisms including *C. elegans* (Zhang *et al.*, 2015). The advantage of the system is that after introducing the AID sequence into the sequence of the studied gene by CRISPR/Cas9 editing, we can use different promoters to achieve local expression of the TIR F-box protein in tissue or cells of interest and thus control the degradation of our protein in space. We can also apply auxin at different time points during development and thus control the degradation in time. The disadvantage of the system is that in comparison to other methods used in this thesis, AID is much more labour intensive.

As the AID system was a new method in our laboratory, it first needed to be optimized. We did not have a *csnk-1* allele tagged with AID available, for the purpose of optimization we therefore used an allele of *mig-5/Dishevelled* tagged with AID (Heppert *et al.*, 2018). Mutation in *mig-5* leads to 100% penetrant QL phenotype (Walston *et al.*, 2006). We first tested degradation of MIG-5 by ubiquitous TIR1 expression from an *eft-3* promoter (Zhang *et al.*, 2015). In all conditions tested we achieved approximately 70% penetrant QL migration defect. This means that we successfully depleted MIG-5 but probably not completely and in some animals, sufficient amount of MIG-5 remained to relay the Wnt signal. It was previously shown that low levels of MIG-5 are sufficient for correct QL migration (Walston *et al.*, 2006). The step in which the QL neuroblast is sensitive to the Wnt signal takes place early during the development of L1 larva, because 4 hours after hatching QL is already dividing (Ou and Vale, 2009). It is therefore also possible that putting newly hatched L1 larvae on auxin is too late and there is not enough time for MIG-5 to be degraded. However, this does not seem to be the case. We also tried to start inducing MIG-5 degradation already in L4 larvae and tested the QL phenotype in the

progeny of these animals and obtained the same penetrance of the QL phenotype as when treating L1 larvae. This suggests that the speed of MIG-5 degradation is not a problem. Indeed, previous work claimed that protein degradation can be achieved within 45 minutes (Zhang *et al.*, 2015).

Next, we tried to deplete MIG-5 by expressing TIR1 in either Wnt producing or Wnt receiving cells using *egl-20* or *egl-17* promoters, respectively. TIR1 expression from *egl-20* promoter was used as a control and no effect on the QL migration was expected. This was indeed the case and we never observed any QL phenotype in any of the experiments. Unfortunately, we also did not obtain any animals with QL phenotype when expressing TIR1 from *egl-17* promoter. This observation could be explained by low expression of TIR1 in the Q neuroblast and therefore by insufficient depletion of MIG-5. However, this would be in conflict with several published works where *egl-17* promoter was successfully used for expression in the Q lineage (Ou and Vale, 2009; Mentink *et al.*, 2014; Shen *et al.*, 2014). Taking into account also our results with CSNK-1 overexpression using the same promoter, we think the most likely explanation of this result is that our version of the *egl-17* promoter is missing some element required for expression in the Q lineage.

In conclusion, although at this point it did not help us to reveal the function of CSNK-1, the AID system could be very powerful tool for protein depletion in *C. elegans* after optimisation of conditions. It has multiple benefits in comparison to other methods we used. The first one is possibility to localize depletion to particular tissue or cell, what we simply cannot reach with RNAi by feeding. Depletion can be also specific in time and due to auxin induction reversible, opening space for conditional knock-down of proteins necessary for development otherwise embryonically lethal.

There are few disadvantages, most notably more preparation work before the experiment can be done. In our experiments the depletion of protein also did not seem complete, but compared to the efficiency of RNAi experiments, the results are still promising and outweigh those handicaps.

6 Literature

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